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(71) Applicants: UNIVERSITY OF MIAMI [US/US]; 1400 N.W. 10th Avenue, Miami, FL 33101 (US). UNIVERSITY TECHNOLOGY CORPORATION [US/US]; Suite 250, 3101 Iris Avenue, Boulder, CO 80301 (US).

(72) Inventors: KRAMER, Richard, H.; 5 Island Avenue #3C, Miami Beach, FL 33139 (US). KARPEN, Jeffrey, W.; University of Colorado Medical School, Dept. of Physiology and Biophysics, C240, 4200 East 9th Avenue, Denver, CO 80262 (US).

(74) Agents: LESTER, Michelle, N. et al.; Pillsbury Madison & Sutro, LLP, 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(57) Abstract

Multimeric tethered ligands are disclosed, comprising a plurality of ligand moieties tethered to a joint moiety, wherein each ligand moiety comprises a ligand for at least one receptor protein having multiple binding sites for the ligand and each ligand moiety is linked by a tether moiety to that joint moiety. Exemplary dimeric tethered ligands are disclosed in which the tether is a simple polymer, polyethylene glycol, which are therefore called "polymer–linked dimers" (PLDs). Exemplary PLDs containing two guanosine 3', 5' cyclic monophosphate (cGMP) moieties are described which are up to 1,000–fold more potent than cGMP in activating cyclic nucleotide–gated (CNG) channels and protein kinases. Each protein responds optimally to a PLD with a different average polymer length, indicating that each has a unique spacing of binding sites. Since optimal ligands are selected empirically, the multivalent tethered ligand strategy allows identification of highly potent and specific agents with no prior structural information about target proteins.

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MULTIMERIC TETHERED LIGANDS AND THEIR USE IN RECEPTOR-LIGAND INTERACTIONS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to multimeric tethered ligands for receptor proteins which have multiple ligand binding sites. More in particular, the present invention relates to compounds comprising two or more ligands for a receptor protein having two or more ligand binding sites, in which the ligands are coupled to each other by a flexible tether, such as a polymer, having an average length which facilitates simultaneous occupancy of each of the multiple ligand binding sites on the receptor protein by an individual ligand of the multimeric ligand. The multimeric tethered ligands of the invention, therefore, provide extraordinarily potent ligands, either agonists or antagonists, which may be used, for instance, therapeutically to target a desired receptor protein with multiple binding sites. The invention also relates to methods for producing such potent multimeric ligands specific for a receptor with multiple ligand binding sites, including ligands specific for a particular member of a family of receptors for a common ligand, without a priori knowledge of the structure of the particular receptor. According to this method, a multimeric ligand selective for a particular receptor is obtained by varying the average length of the tether connecting multiple ligands to determine an optimal length for simultaneous binding of individual ligands to multiple binding sites separated by the particular distance between binding sites occurring in that particular receptor protein.

2. Description of Related Art

A variety of biological receptor proteins are known to have multiple ligand binding sites, either on an individual protein subunit or on multiple subunits. For instance, cyclic nucleotides are ubiquitous intracellular messengers that affect myriad cellular functions. Cyclic nucleotide effectors include CNG ion channels, such as those from vertebrate photoreceptors and olfactory neurons (1), and protein kinases activated either by cGMP (2) or by adenosine 3',5'-cyclic monophosphate (cAMP) (3) (PKG and

PKA, respectively). Each of these proteins contains 4 cyclic nucleotide binding sites, either on individual subunits, such as in CNG channels (4-6), or grouped two to a subunit, such as in PKA and PKG (2,3). These binding sites all share considerable amino acid sequence homology (7), but they differ in their apparent binding affinities for different cyclic nucleotides and their derivatives. In addition, the extent to which binding of a particular derivative causes functional activation also varies between the different proteins. These differences have led to the identification of cyclic nucleotide derivatives that show some selectivity as agonists or antagonists for individual CNG channels or cyclic nucleotide-dependent protein kinases (8). However, there remains a need for ligands which show greater affinity and selectivity for a particular cyclic nucleotide-binding protein or for an individual representative of any other receptor family sharing a common ligand for which each receptor in the family has multiple ligand binding sites.

Multivalent receptor binding molecules are known in the art. For instance, cross-linked antibodies are known to cause individual surface immunoglobulin receptors on B-lymphocytes to form aggregates known as patches, which collect into a cap assembly at one pole of the cell. See, for example, Graziadei, L. et al, *Nature 347*:396-399 (1990). Capping is thought to be an essential event in antibody-mediated signal transduction across cell membranes.

In addition, certain polypeptide growth factors and hormones are known to function as bivalent ligands to effect receptor dimerization. For example, human growth hormone molecules each have two binding sites, enabling the hormone to bind sequentially two separate receptor proteins. Similarly, receptor dimerization of EGF receptor family tyrosine kinases is mediated by the bivalent behavior of at least one ligand. Also, a differentiation factor encoded by the *neu* gene is known to simultaneously bind two receptor proteins, creating functional dimers that mediate signal transduction, thereby promoting cell growth. See, for example, Tzahar, E. et al., EMBO J. 16:4938-4950 (1997).

Analogs of small molecule ligands which comprise more than one moiety structurally related to such a ligand are also known. For instance, analogs of acetylcholine are known in which two identical moieties structurally related to acetylcholine are connected by a short methylene polymer, for instance, decamethonium bromide in which

two trimethylamonium bromide residues are connected by a ten carbon polymeric chain to produce the following structure:

$$Br(CH_3)_3N\text{-}(CH_2)_{10}\text{-}N(CH_3)_3Br.$$

See, for example, del Castillo, J. and Katz, B., Proc. Royal Soc. London B, *146*:369-381 (1957). However, there is no indication in the art that such a dimeric analog was intended or would be able to bind to multiple binding sites on any receptor protein. Thus, the short length of the ten carbon polymer between the two trimethylamonium bromide residues appears to be incompatible with simultaneous occupancy of multiple binding sites on a receptor protein, such as an acetylcholine receptor.

The concept of a "tethered ligand" has been used to describe a portion of a receptor protein which binds to a ligand binding site of that same receptor protein. For instance, such a receptor is thought to be involved in thrombin inhibition of platelet adenylate cyclase. Seiler, S. M. et al., Biochem. Biophy. Res. Comm. *182*:1296-1302 (1992). A portion of the receptor sequence resembles the carboxy terminus of hirudin, an peptide inhibitor of thrombin, and thrombin evidently recognizes this sequence and cleaves a single peptide bond in the receptor protein that releases a peptide containing the original N-terminus. The resulting new N-terminus apparently serves as a tethered ligand which activates the receptor.

The term "tethered ligand" also has been used to describe a ligand attached by a polymer to a macromolecule or molecular complex, for instance, liposomes, to target the macromolecule or complex to a receptor for the ligand. Attaching a ligand to such a macromolecule or complex by a flexible tether is known to influence the binding of the ligand to its receptor. For example, in a model system involving binding of avidin with an immobilized avidin-binding protein, streptavidin, in which the biotin was tethered to an immobilized lipid layer by polyethylene glycol (PEG), it was shown that the addition of the flexible molecular tether extends the effective range of a specific ligand-receptor interaction by the length of the fully extended chain, and thus that the effective receptor-ligand on-rate is controlled by the tether length and dynamics. Wong, J. Y., et al., *Science 275*:820-822 (1997). Other systems have been used to anchor multiple copies of a ligand to a particle using a polymeric linkage. For instance, bacteriophage particles displaying multiple copies of peptide ligands, called "phage-tethered peptides," have been used as

multivalent ligands to "stain" and monitor expression of various receptor proteins. Li. M., *Nature Biotechnology 15*:559-563 (1997).

Recently, a nuclear magnetic resonance (NMR) method for discovering high-affinity ligands for proteins, called "Structure-Activity Relationships (SAR) by NMR," has been described. Shukar, S. et al., *Science 274*:1531-1534 (1996). The technique is a linked-fragment approach wherein ligands are constructed from building blocks that have been optimized for binding to individual protein subsites (within a single binding site for a known ligand). When two "lead" fragments have been selected, their location and orientation in the ternary complex are determined experimentally, either by NMR spectroscopy or x-ray crystallography. On the basis of this structural information, compounds are synthesized in which the two fragments are linked together, with the goal of producing a high affinity ligand. *Id.* at page 1532. In one example, compounds with nanomolar affinities for the FK506 binding protein (FKBP) were rapidly discovered by tethering two ligands with micromolar affinities. This protein forms a complex with the potent immunosuppressant FK506. Many compounds were found that bound weakly to FKBP. One particular pipecolinic acid derivative ("2") showed the highest affinity for FKBP, and the binding site for this molecule on FKPB was shown to be the same as that of a pipecolinic acid moiety of FK506, by NMR analyses. To identify molecules that interact with FKBP at a nearby site, a library of compounds was screened for binding in the presence of saturating amounts of the pipecolinic acid derivative 2. A benzanilide derivative was found to bind at a site near the pipecolinic acid derivative 2, and a further derivative ("9") of this compound served as the best ligand for this second site. A model of the ternary complex of derivatives 2 and 9 and FKBP was generated on the basis of NMR studies. *Id.* at page 1533. In this model, the methyl ester of derivative 2 is close to the hydroxyl group on the benzoyl ring of derivative 9. Linkers were then designed that would attach to these groups, span the distance between the two fragments, and having no steric clashes with the protein. Linker chains of three to six carbons were tested, and maximum affinity of the linked fragments was a chain of three carbon atoms.

While the above approach may expedite design of high affinity ligands for a single ligand binding site, such as the binding site for FK506 on the FK506 binding protein, by identifying small molecules which bind to different subsites with that single ligand

binding site, it does not specifically address design of high affinity ligands for receptors with multiple binding sites for the same ligand, much less ligands which would discriminate among related receptors that bind a common ligand at multiple binding sites, such as cyclic nucleotide effectors.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide ligands having high affinity for receptors with multiple ligand binding sites, particularly ligands which would discriminate among related receptors that bind a common ligand at multiple binding sites, such as cyclic nucleotide effectors. In particular, it is an object of the invention to provide tethered multimeric ligands in which multiple ligands for a receptor protein with multiple ligand binding sites are connected by a flexible tether of a length which provides simultaneous occupancy of multiple binding sites by individual ligand moieties in the multimeric ligand. In these multimeric tethered ligands, the length may be optimized to provide simultaneous occupancy of multiple binding sites separated by a particular distance which occurs in a particular member of a group of related receptors that bind a common ligand at multiple binding sites

According to one aspect of the invention, therefore, there is provided a compound which is a multimeric tethered ligand comprising a plurality of ligand moieties tethered to a joint moiety, wherein each ligand moiety comprises a ligand for at least one receptor protein having multiple binding sites for said ligand and each ligand moiety is linked by a tether moiety to that joint moiety. This compound has a structure which may be described by the general formula:

$$J(C_J^1\text{-}T^1\text{-}C_L^1\text{-}L^1)(C_J^2\text{-}T^2\text{-}C_L^2\text{-}L^2)(C_J^3\text{-}T^3\text{-}C_L^3\text{-}L^3)\ldots(C_J^x\text{-}T^x\text{-}C_L^x\text{-}L^x)$$

wherein:

x is the number of ligand moieties in said compound and is an integer greater than or equal to 2;

each of L^1 to L^x is one of said ligand moieties and comprises a ligand which is the same as or different from the ligand of any other ligand moiety in said compound,

J is said joint moiety selected from the group consisting of a covalent bond, an atom and a molecule;

each of C_J^1 to C_J^x is a joint coupling moiety selected from the group consisting of a covalent bond, an atom and a molecule, and is covalently coupled to said joint moiety;

each of C_L^1 to C_L^x is a ligand coupling moiety selected from the group consisting of a covalent bond, an atom and a molecule, and is covalently coupled to one of said ligand moieties, L^1 to L^x, respectively;

each of T^1 to T^x is a tether moiety and is a linear polymeric molecule having one end covalently coupled to said joint coupling moiety,C_J^1 to C_J^x, respectively, and the other end covalently coupled to a ligand coupler moiety, C_L^1 to C_L^x, respectively, thereby linking said ligand moiety to said joint moiety; and

each ligand moiety, L^1 to L^x, and tether moiety coupled thereto, T^1 to T^x, respectively, are not both peptides and are not both polynucleotides.

In preferred embodiments of this compound, x = 2 and each of L^1 and L^2 comprises a ligand for the same receptor protein. Such a dimeric tethered ligand of the invention may be described by a simpler version of the above formula, as follows:

$$L^1\text{-}C_L^1\text{-}T^1\text{-}C_J^1\text{-}J\text{-}C_J^2\text{-}T^2\text{-}C_L^2\text{-}L^2.$$

In a more preferred embodiment of this dimeric compound, each of L^1 and L^2 comprises the same ligand.

In preferred embodiments, each of the tethers of the invention compounds, T^1 and T^2, is a polymeric molecule selected from the group consisting of polymers containing only C, H and O, polynucleotides and polypeptides. In highly preferred embodiments of the compound, C_J^1,T^1, C_J^2 and T^2 together are a molecule of polyethylene glycol (PEG) having an average molecular weight in the range of about 47 to about 100,000 daltons. Particularly preferred are PEG molecules having an average molecular weight in the range of about 282 to about 20,000 daltons. Other reasonably flexible polymers may be used to form the tether of the invention molecule, provided that the average (root mean square; "rms") length of the tether is sufficient to span the distance between two ligand binding sites on the target receptor, typically on the order of about 10 to about 100 angstroms (Å).

For instance, Stayton, P. S., et al., (*Nature 378*:472-474 (1995)) have described stimuli-response (for instance, temperature-sensitive) polymers of poly N(isopropylacrylamide) for control of protein-ligand recognition which may be adapted to produce the multimeric ligands of the invention.

The ligand moieties, for instance L^1 and L^2, of the multimeric ligands of the invention may comprise ligands for any ligand binding protein, including such ligands as compounds comprising a purine or a nucleoside or a nucleotide, including cyclic nucleotides and oligonucleotides, compounds comprising an amino acid, such as peptides, polypeptides and proteins which are know to function as hormones, growth factors, cytokines or anti-idiotypic antibodies, for instance. Ligand moieties of the invention may also comprise various small molecule ligands, such as drugs and metabolites.

In particularly preferred embodiments of the compound of the invention, each of L^1 and L^2 is a guanosine 3',5'cyclic monophosphate (cGMP) molecule or a derivative thereof, and each of $C_L{}^1$- and $C_L{}^2$-has the structure: S-CH_2-C H_2-SO_2- and is covalently coupled to the carbon in the 8 position of the guanosine moiety in the cGMP molecule or derivative thereof. In these compounds, L^1 and L^2 are ligands for a receptor protein selected from the group consisting of a cyclic nucleotide-gated ion channel or a guanosine 3',5'-cyclic monophosphate activated kinase. As described below, exemplary compounds of the invention exhibit an affinity for such receptors that is substantially greater than the average affinity of a monomer of the ligand for that receptor. Thus, compounds of the invention may exhibit an affinity for a receptor with multiple ligand binding sites that is at least about 10 times, preferably at least about 100 times to at least about 2,000 times, and still more preferably at least about 10,000 times greater than the average affinity of a monomer of the ligand for that same receptor.

Other ligands particularly suited for use in the multimeric tethered ligands of the invention include the following ligands or derivatives thereof, which are listed in relation to the receptor(s) to which they bind:

1. LIGAND-GATED ION CHANNELS

Agonists and antagonists for ligand-gated channels. General reviews: *Receptor and Ion channels Nomenclature Supplement* (S.P.H. Alexander and J.A. Peters, eds.) Trends in Pharmacological Sciences; Elsevier, Cambridge, UK (1997); *Handbook of Receptors and*

Channels:.Ligand and Voltage-Gated Ion Channels (R.A. North, ed) CRC Press (1994). All ligands listed apparently bind to multiple sites on a given receptor protein.

A. **Nicotinic acetylcholine receptors.** Agonists and antagonists of muscle and neuronal nicotinic acetylcholine receptors. Includes α-bungarotoxin, neuronal bungarotoxin, anatoxin, ABT418, cytisine, epibatidine, nicotine, atropine, (+)-tubocurarine, and other agonists and antagonists (see Lindstrom J.M., (1994) in *Handbook of Receptors and Channels:.Ligand and Voltage-Gated Ion Channels* (R.A. North, ed) CRC Press, pp. 153-175.

B. **Capsaicin receptors** Capsaicin, capsazepine, ruthenium red, resiniferatoxin, and their derivatives. (Caterina et al., 1997).

C. **Cyclic nucleotide-gated channels.** Cyclic nucleotides (cAMP, cGMP, cCMP, cIMP, cUMP) and 8-substitued derivatives including, but not limited to 8-Br cAMP and cGMP, 8-parachlorothio (8-pCPT) cAMP and cGMP, 8-fluorosceinyl cAMP and cGMP, and 8-n-propylthio cGMP. Also included are phosphorothioate derivatives of cAMP, cGMP, and their 8-substituted derivatives , partially listed above. Both isomers of these phosphorothioate derivatives are included (for example Rp-cAMPS and Rp-cGMPS, Sp-cAMPS, Sp-cGMPS, Rp- and Sp-8-pCPT-cAMPS and Rp- and Sp-8-pCPT-cGMPS. Also included are PET (β-phenyl-1, N2-etheneoguanosine-3',5'-cyclic monophosphate) derivatives of cAMP and cGMP, PET forms of 8-substituted derivatives of cAMP and cGMP, and PET forms of phosphorothioate derivatives of cAMP and cGMP. (Botelho et al., 1988; Brown et al., 1993; Butt et al., 1990, 1994, 1995; Kramer and Tibbs, 1996; Wei et al., 1996; Zimmerman et al., 1985)

D. **Ionotropic GABA receptors (GABAA and GABAC receptors).** Agonists and antagonists of the GABA, benzodiazepine, and barbiturate binding sites on GABA receptors. GABA site ligands: Isoguvacine, muscimol, THIP, piperidine-4-sulphonic acid, bicuculline, SR95531, and their derivatives and related compounds. Benzodiazepine site: benzodiazepines including flunitrazem, diazepam, zolpidem, abecarnil, ZK93423, DMCM, Ro194603, flumazenil, ZK93426, CGS8216, cis-4-aminocrotonic acid and related compounds and derivatives. Barbiturate site: phenobarbitoal, pentabarbitol, and related compounds and derivatives. Borman and

Feigenspan (1995), Bowery, (1997), Johnston (1996), Sieghart (1995), Whiting et al., (1995).

E. **Ionotropic glutamate receptors**

NMDA: Agonists and antagonists of the glutamate binding sites including, but not limited to glutamate, aspartate, N-methyl D-aspartate (NMDA), D-AP5, D,L(tetrazol-5-yl)glycine, CGS19755, CGP37849, LY233053, CPP, dizocilpine and related compounds and derivatives. Agonists and antagonists of the glycine-binding site including, but not limited to glycine, (+)HA966, D-serine, 5,7-dichlorokynurenate, L689560, MNQX and related compounds and derivatives.

AMPA: Agonists and antagonists including, but not limited to AMPA, (s)-5-flurowillardine, NBQX, CNQX, LY215490, LY293558, GYK153655, and related compounds and derivatives.

Kainate: Agonists and antagonists including, but not limited to kainate, quisqualate, 4-methyl glutamic acid, domoic acid, NS102 and related compounds and derivatives.

References: Bettler and Mulle, (1995); Fletcher and Lodge, (1996); Hollman and Heinemann, (1994); Sucher et al., (1996); Wo and Oswald (1996).

F. **Glycine receptors** Agonists and antagonists including glycine, β-alanine, taurine, strychnine, picrotoxin, quinoline, cyanotriphenylborate, their derivatives and related compounds. Becker, (1992); Schmeiden and Betz, (1995); Rundstrom et al., (1994); Kuhse, 1995

G. **Ionotropic purinergic receptors** (P_{2X} receptors) ATP, ADP, AMP, and their analogs and derivatives, particularly ATP-γ-S and α,β methylene-ATP. Also GTP, GDP, GMP, and their analogs and derivatives, particularly GTP-γ-S. Suramin, an antagonist of P_{2x} receptors. Also adenosine, guanosine, cytosine, and inosine, and their analogs, derivatives and related compounds. (Brake et al., 1994; Burnstock, 1990; Cusack and Hourani, 1990; Dalziel and Westfall, 1994; Fredholm et al., 1994).

H. **Ionotropic serotonin** receptors ($5\text{-}HT_3$ receptors) Agonists and antagonists including 5-hydroxytryptamine (5-HT, also known as serotonin), 2-Methyl-5-HT, m-CPBG, granisetron, ondansetron, tropisteron, zacopride, α-methyl-5-HT, and

derivatives of above agents. (References in TIPS review book on Receptors and Ion channels , 1997).

2. OTHER MULTI-SUBUNIT ION CHANNELS REGULATED BY LIGANDS

A. K_{ATP} channels (ATP, ADP, AMP and other nucleotides and derivatives, also sulfonylurea compounds and derivatives)

B. IP_3 receptors. Ligands include $!P_3$, IP_4 and other inositol phosphates, and derivatives.

3. TRANSPORT PROTEINS

Proteins in this group include "ABC" transporters (e.g. multidrug resistance protein, cyctic fibrosis transmembrane regulator (CFTR) protein, and adenylate cyclase). There is evidence that at least some of these protein kinase have multiple nucleotide binding sites that are not catalytic, but rather operate to regulate transport activity. Therefore, PLD ligands for this group of proteins include ATP, GTP, CTP, ADP, GDP, AMP, GMP, cAMP, cGMP, cCMP, NADP, NADPH, cyclic ADP-ribose, and derivatives and analogs of these and other nucleotides.

4. OTHER ALLOSTERIC PROTEINS. A wide variety of enzymes, involved in metabolism, signaling, and more specialized functions, such as muscle contraction, are regulated in an allosteric manner by the binding of regulatory ligands to multiple binding sites. Nucleotides are the most common of these regulatory ligands, so PLD ligands for this group of proteins include ATP, GTP, CTP, ADP, GDP, AMP, GMP, cAMP, cGMP, cCMP, NADP, NADPH, cyclic ADP-ribose, and derivatives and analogs of these and other nucleotides. The following enzymes are examples: aspartate carbamoyl transferase, glycogen phosphorylase, cyclic AMP- and cyclic GMP-dependent proteins kinases, and phosphofructokinase. This last enzyme appears to multiple regulatory sites that bind GDP; the effect of GDP on enzynmatic activity has a Hill coefficient near 2. References for allosteric regulation of enzymes include: Fersht, A. (1985) *Enzyme Structure and Mechanism* (2^{nd} edn.), W.H. Freeman and Co., NY; Perutz, M. (1990) *Mechanisms of Cooperativity and Allosteric Regulation in Proteins* ; Cambridge Univ. Press.

References cited in the above list

Becker, C-M (1992) in *Handbook of Experimental Pharmacology, Selective Neurotoxicity* (Vol. 102) (Herken, H. and Hucho, F., eds.), pp539-575, Springer-Verlag.

Bettler, B. and Mulle, C. (1995). *Neuropharmacol.* 34:123-139.

Botelho L.H.P., Rothermael, J.D., Coombs, R.V., and Jastorff, B. (1988). *Methods Enzymol.* 159:159-172.

Bormann, J. and Feigenspan, A. (1995). *Trends Neurosci.* 18:515-519.

Bowery, N.G. (1997). In *The GABA Receptor* (2nd edn.) (Enna, S.J. and Bowery, N.G., eds.), pp. 203-236. Humana Press.

Brake, A.J., Wagenbach, M.L., and Julius, D.J. (1994). *Nature* 371:519-523.

Brown, R.L., Bert, R..J., Evans, F.E., and Karpen, J.W. (1993). *Biochem.* 32:10089-10095.

Burnstock, G. (1990). *Ann. N.Y. Acad. Sci.* 603:1-18

Butt, E., Van Bemmelen, M., Fischer, L., Walter, U., and Jastorff, B. (1990). *FEB Letters.* 263:47-50.

Butt, E., Eigenthaler, M., and Genieser, H.G. (1994). *Eur. J. Pharmacol.* 269:265-268.

Butt, E., Pohler, D., Genieser, H.G., Huggins, J.P., and Bucher, B. (1995). *Br. J. Pharmacol.* 116:3110-3116.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). *Nature* 389:816-824.

Cusack, N.J. and Hourani, S.M.O. (1990). *Ann. N.Y. Acad. Sci.* 603:172-181.

Dalziel, H.H. and Westfall, D.P. (1994). *Pharm. Rev.* 46:449-464.

Fletcher, E.J. and Lodge, D. (1996). *Pharmacol Ther.* 70:65-89.

Fredholm, B.B. et al., (1994). *Pharm. Rev.* 46:143-156.

Hollman, M. and Heinemann, S. (1994). *Annu. Rev. Neurosci.* 17:31-108.

Johnston, G.A.R. (1996). *Pharmacol. Ther.* 67:187-246.

Kramer, R.H. and Tibbs, G.R. (1996). *J. Neurosci.* 16:1285-1293.

Kuhse, J. et al., (1995). *Curr Opin Neurobiol.* 5:318-323.

Rundstrom, N., et al., (1994). *Proc. Natn. Acad. Sci. USA* 91:8950-8954.

Schmeiden, V. and Betz, H. (1995). *Mol. Pharmacol.* 48:919-927.

Sieghart, W. (1995). *Pharmacol Rev.* 47:182-234.

Sucher, N.J. et al., (1996). *Trends Pharmacol. Sci.* 17:348-355.

Suprenant, A. et al. (1995). *Trends Neurosci.. 18*:224-229.

Wei, J-Y., Cohen, E.D., Yan, Y-Y., Genieser, H-G., and Barnstable, C.J. (1996). *Biochem.* 35:16815-16823

Whiting, P.J. et al., (1995). *Int. Rev. Pharmacol.* 38:95-138.

Wo, Z.G. and Oswald, R.E. (1996). *Trends Neurosci.* 18:161-167.

Zimmerman, A. L., Yamanaka, G., Eckstein, F., Baylor, D.A., and Stryer, L. (1985). *Proc. Natn. Acad. Sci. USA* 82:8813-8817.

A derivative of a ligand in the context of the present invention indicates a moiety of an invention compound which is substantially structurally similar to the ligand and retains an ability to bind specifically to a receptor protein having a binding site that specifically binds to that ligand.

In another aspect, the invention provides a method of activating or antagonizing activation of a receptor protein comprising multiple binding sites for a ligand L comprising adding to the receptor protein, under conditions where the ligand L activates or antagonizes activation of the receptor protein, a compound of the invention in which each of said ligand moieties L^1 to L^x comprises said ligand L or a derivative thereof, and the average (rms) length of the portion of said compound linking any two of said ligand moieties L^1 to L^x is greater than or equal to the distance between any two of said multiple binding sites, whereby each of at least two of the ligand moieties L^1 to L^x binds to one of the multiple binding sites on the receptor protein, thereby activating or antagonizing activation of the receptor protein. In the present context, the average (root-mean-squared; rms) length of a particular polymer preparation may be determined empirically, as in previous studies which have determined the rms lengths of several specific PEGs (12) of known molecular weights, which, like other flexible polymers, increases with the square root of the number of monomeric units (13). This information then allows estimation of the rms length of various preparations of the same polymer, based on the determination of the average molecular weight of the polymer preparation.

Another aspect of the invention is a method of obtaining a compound invention which activates or antagonizes activation of a receptor protein comprising multiple binding sites for a ligand L, said compound having a higher specific activity than said ligand L for activation of said receptor protein, said method comprising: (a) providing a group of compounds of the invention, each compound in the group having each of the ligand moieties L^1 to L^x comprising ligand L or a derivative thereof. Each compound in said group differs in the average (rms) length of the portion of the compound (that is, the flexible tether portion) linking any two of said ligand moieties L^1 to L^x, the lengths of

these portions in the molecules being selected to span the range of possible distances between any two of the multiple binding sites on the receptor protein. The method further comprises (b) adding a fixed amount of each compound in the group separately to the receptor protein, under conditions where the ligand L activates or antagonizes activation of the receptor protein, (c) determining the level of activation or antagonization of activation of the receptor protein produced by that fixed amount of each compound in that group, (d) comparing the level of activation or antagonization of activation of the receptor protein produced by the fixed amount of each compound in the group to the level of activation or antagonization of activation obtained by adding the same fixed amount of the ligand L to the receptor under the same conditions, and (e) selecting the compound in the group producing the highest level of activation or antagonization of activation of the receptor protein that is greater than the level of activation or antagonization of activation of the receptor by the ligand L.

Yet another aspect of the invention relates to a method of determining whether a receptor protein activated or antagonized by a ligand L comprises multiple binding sites for the ligand L such that binding of the ligand L to more than one of the multiple binding sites activates or antagonizes activation of the receptor protein to a higher level than does occupation of only one of those multiple binding sites. This method comprises: (a) providing a group of compounds of the invention, each compound in the group having each of the ligand moieties L^1 to L^x comprising the ligand L or a derivative thereof, and each compound in the group differing in the average (rms) length of the portion of the compound linking any two of the ligand moieties L^1 to L^x. The lengths of these portions in the molecules are selected to span the expected range of possible distances between any two of the multiple binding sites on the receptor protein. This method further comprises: (b) adding a fixed amount of each compound in the group separately to the receptor protein, under conditions where the ligand L activates or antagonizes activation of the receptor protein, and (c) determining whether any compound in the group produces a higher level of activation or antagonization of activation of said receptor protein than produced by adding the same fixed amount of said ligand L to said receptor under the same conditions. In this method, in a higher level of activation or antagonization of activation of the receptor protein by a compound in the group compared to ligand L

indicates that the receptor protein activated by ligand L comprises multiple binding sites for that ligand L such that binding of said ligand L to more than one of the multiple binding sites activates said receptor protein to a higher level than does occupation of only one of the multiple binding sites.

More particularly, as described below, when the rms length of the tether between two ligand moieties comprising ligand L is substantially less than the distance between two binding sites for ligand L on the receptor protein, a dimeric ligand of the invention is expected to exhibit an apparent affinity for the receptor about twice that of the monomeric ligand L alone. As the rms length of the flexible tether between two ligand moieties comprising ligand L is increased, the apparent affinity of the dimeric ligand will substantially increase, reaching a maximum level when the rms length is approximately equal to the distance between the two binding sites on the receptor which bind ligand L. Longer tethers beyond that optimum length approximately equal to the distance between the two binding sites for the ligand L on the receptor results in reduced apparent affinity of the dimeric ligand of the invention. In this way, the rms length of the flexible tether of the dimeric ligand of the invention can be optimized so as to selectively bind to a receptor having a particular distance between two binding sites for ligand L. Similarly, the rms lengths of tethers connecting more than two ligand moieties can be optimized for binding to a protein having more than two binding sites for the same ligand moieties, by a similar optimization of rms tether lengths between a pair of ligand moieties whereby multiple peaks of high affinity are expected at rms tether lengths approximately equal to each distance separating any pair of binding sites on the receptor.

The compounds of the invention are useful, for instance, for studying the structure of receptors with multiple ligand binding sites, and for therapeutic modulation of receptor-mediated activities. For example, cyclic nucleotide dependent kinases have been implicated in a wide variety of functions including plasticity of growth and neuronal connections, skeletal muscle contraction, regulation of cardiac rate and output, kidney and liver function, secretion from endocrine and exocrine glands, and many more bodily prociesses. Cyclic AMP-dependent protein kinases have more than 200 known phosphorylation targets in cells. Despite the widespread importance of these molecules in cell signalling processes, selective ligands for these proteins have been lacking. Many

present drugs affect cAMP signaling by targeting membrane receptors, such as the beta-adrenergic receptor (targeted by propanolol), involved in cyclic nucleotide-signaling cascades in a variety to cell types, or enzymes involved in cyclic nucleotide metabolism, such as phosphodiesterase (inhibited, for instance, by theophylline). For cGMP, many drugs target cGMP production important for regulating vasodilation and kidney function, such as nitroglycerin and sodium nitroprusside. The present cyclic nucleotide multimeric tethered ligands may be used to directly and specifically target either the cyclic nucleotide-dependent kinases or the CNG channels for pharmacological intervention, rather than targeting upstream components of the signaling cascade, thereby providing drugs which may act at lower doses, more effectively, and with fewer side effects.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a preferred compound of the invention, a polymer-linked ligand dimer (PLD). Panel A. Structure of a PLD containing 2 cGMP moieties and a polyethylene glycol (PEG) linker, with n ethylene glycol units. Panel B. Schematic diagrams of PLDs binding to a channel with 4 ligand binding sites. Illustrated are PLDs with polymers that have an average length that is too short (left), just right (center), or longer than necessary (right), to allow the ligands to span two binding sites on the channel. In the optimal PLD, once one ligand binds, the effective concentration (C_{eff}) of the tethered unbound ligand is calculated from the volume of the hemisphere with radius r in cm) circumscribed by the tethered molecule. Thus, $C_{eff} = 1{,}000/(N_A*2/3\pi r^3)$, where N_A is Avagodro's number.

Figure 2 depicts activation of CNG channels by PLDs and monomeric cyclic nucleotides. Dose-response curves of activation of OLF (Panel A) and RET (Panel B) CNG channels by cGMP, 3 different PLDs, and the PLM. Responses from each patch were normalized to the response elicited by 2 mM cGMP. Fits to the Hill equation yield $K_{1/2}$ and n values for RET channels as follows: cGMP: $K_{1/2} = 72$ μM, n = 2.0; 282 PEG-(cGMP): $K_{1/2} = 11$ μM, n = 1.3; 3,400 PRG-(cGMP)2: $K_{1/2} = 4.7$ μM; n = 0.9; 20,000 PEG-(CGMP)2: $K_{1/2} > 75$ μM. K1/2 and n values for OLF channels are described elsewhere herein.

Figure 3 shows dissociation of PLDs from CNG channels. Panel A. Tail currents reflecting dissociation of cGMP and various PLDs. Free agonists were applied during the indicated period, and washed away with continuous perfusion of control saline. Panel B. Effect of free cGMP on the dissociation of bound PLDs. CNG channels were "loaded" with 2,000 PEG-(cGMP)2 for 15 sec. Subsequently, during the "chase" period, free PLD was removed and replaced with various concentrations of cGMP. Finally, the patch was perfused with control saline to examine the degree to which PLDs had dissociated. Kinetic experiments in A and B were performed with OLF channels.

Figure 4 illustrates the identification of optimal PLDs for cGMP-binding proteins according to the method of the invention. Panel A. Activation of CNG channels (RET and OLF). Panel B. Activation of PKG. Arrows indicate PLDs optimal for activating each protein. Polymer lengths were estimated from previous determinations (12), assuming an increase with the square root of MW (13).

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention relates to multimeric tethered ligands comprising a plurality of ligand moieties tethered to a joint moiety, wherein each ligand moiety comprises a ligand for at least one receptor protein having multiple binding sites for said ligand and each ligand moiety is linked by a tether moiety to that joint moiety. This compound has a structure described by the general formula:

$$J(C_J^1\text{-}T^1\text{-}C_L^1\text{-}L^1)(C_J^2\text{-}T^2\text{-}C_L^2\text{-}L^2)(C_J^3\text{-}T^3\text{-}C_L^3\text{-}L^3)\ldots(C_J^x\text{-}T^x\text{-}C_L^x\text{-}L^x)$$

wherein each of the variables is defined as hereinabove. The invention is based on observations made with dimeric embodiments generally described by the formula $L^1\text{-}C_L^1\text{-}T^1\text{-}C_J^1\text{-}J\text{-}C_J^2\text{-}T^2\text{-}C_L^2\text{-}L^2$, particularly exemplary dimeric ligands in which the tether is a simple polymer, polyethylene glycol, which are therefore called "polymer-linked dimers" (PLDs).

A leading approach to drug design involves determining the structure of binding sites on proteins, providing a template for constructing new ligands. Alternatively, combinatorial chemistry utilizes random combinations of chemical groups to generate diverse molecules, which are screened to select effective species. The present inventors

devised a third strategy combining elements of these approaches using PLDs in which two ligands are linked with a variable length polymer chain. This strategy can be used to develop extraordinarily potent ligands for proteins with multiple binding sites. Exemplary PLDs containing two guanosine 3', 5' cyclic monophosphate (cGMP) moieties are described below. These PLDs are up to 1,000-fold more potent than cGMP in activating cyclic nucleotide-gated (CNG) channels and protein kinases. Each protein responds optimally to a PLD with a different average polymer length, indicating that each has a unique spacing of binding sites. Since optimal ligands are selected empirically, the multivalent tethered ligand strategy allows identification of highly potent and specific agents with no prior structural information about target proteins.

The multimeric tethered ligand strategy for developing selective and extraordinarily high affinity ligands for proteins that possess multiple ligand binding sites, such as cyclic nucleotide-binding proteins, is illustrated here using PLDs. PLDs were synthesized by reacting a sulfhydryl derivative of cGMP (9) with a bifunctional vinylsulfone-derivatized PEG (10). Alternative flexible polymers and chemistries for bifunctional coupling of such polymers with various ligands are known in the art. The present reaction produces a "barbell-shaped" molecule that contains two cGMPs connected to both ends of a flexible polymer via a thioether linkage at the 8-position of the purine (see Fig. 1A). Previous work showed that conjugation of bulky groups at the 8-position, rather than being detrimental, actually results in an increase in the apparent affinity for CNG channels (9,11). Starting with PEGs of different molecular weights, a series of PLDs were synthesized with different average distances separating the two cGMP moieties. Each PLD was assigned a name on the basis of the molecular weight of the polymer (in this case PEG) and on the nature of the ligand (in this case cGMP). For example, a PLD composed of a 3,400 molecular weight PEG conjugated to 2 cGMPs is denoted "3,400 PEG-$(cGMP)_2$". Previous studies have determined the average (root-mean-squared; rms) lengths of several specific PEGs (12), which, like other flexible polymers, increases with the square root of the number of monomeric units (13). This information allowed estimation of the rms length of each of these PLDs.

Figure 1B schematically illustrates how changing the polymer length of a PLD affect its ability to bind to a protein with multiple ligand binding sites, using the

interaction between a CNG channel and 3 distinct PLDs. Each of the four subunits that comprise the CNG channel contains a cyclic nucleotide binding site that is accessible to the cytoplasmic solution. When the rms length of the PLD is much shorter than the distance between two cyclic nucleotide binding sites on the channel, the apparent affinity of the PLD should be about twice that of cGMP itself. This assumes that the polymer has no effect on the ability of the cGMP moieties either to bind, or to activate the channel. When the rms length of the PLD exactly matches the distance between the binding sites on the channel, a dramatic increase in the apparent affinity is predicted. Consider, for example, a CNG channel in which 39 Å is required to span two binding sites. After the first cGMP binds, the diffusion of its tethered cognate cGMP will be restricted, such that its effective concentration will be 6.7 mM (14). This value is 100-2,000 times higher than the typical apparent affinity ($K_{1/2}$ value) of CNG channels for cGMP. Thus, once one cGMP moiety binds, its cognate cGMP will have a very high probability of binding, greatly increasing the overall affinity of the PLD for the channel. With larger PLDs, whose rms length exceeds the distance between binding sites, the effective concentration of the cognate ligand should decline, reducing the apparent affinity for the channel.

This prediction was tested by applying PLDs, as well as a control "polymer-linked ligand monomer" containing only one cGMP moiety (PLM; ref. 15), onto excised patches containing rat olfactory (OLF), or bovine rod photoreceptor (RET) CNG channels (16). Dose-response curves were fit with the Hill equation to determine the apparent affinity of the channels for each PLD and the PLM. Figure 2A shows that at saturating concentrations, all of these agents activate OLF CNG channels to the same extent as does saturating cGMP itself, indicating that they are all full agonists. The apparent affinity of the PLM ($K_{1/2}$=1.4 mM) was slightly higher than that of cGMP ($K_{1/2}$=3.1 mM). This indicates that conjugation of PEG onto the 8-position of cGMP had a minor beneficial effect on the ability of the cGMP moiety to bind to the OLF channel, and to elicit its activation. As expected, the apparent affinity of the shortest PLD, 282 PEG-$(cGMP)_2$, was somewhat higher ($K_{1/2}$=0.85 mM) than that exhibited by either cGMP or the PLM. The Hill coefficient, indicative of the minimum number of individual molecules required for significant activation, was similar for cGMP, the PLM, and 282 PEG-$(cGMP)_2$ (1.7, 2.0, and 2.0, respectively).

In contrast to the modest enhancement of affinity exhibited by 282 PEG-$(cGMP)_2$, a larger PLD, 3400 PEG-$(cGMP)_2$, had a dramatic effect on the OLF channels. The estimated $K_{1/2}$ for this PLD was 12 nM, 260-fold lower than exhibited by cGMP itself. An even larger PLD, 20,000 PEG-$(cGMP)_2$, had a much less dramatic increase in the apparent affinity (K1/2=250 nM). The Hill coefficient values for 3,400 PEG-(cGMP)2 and 20,000 PEG-$(cGMP)_2$ were 1.1 and 0.8, respectively, about half as large as for the PLM or the smaller 282 PEG-$(cGMP)_2$. This is consistent with the larger, but not the smaller, PLDs simultaneously occupying two binding sites on the channel.

The effect of PLDs on RET channels is similar to that on OLF channels, with the intermediate PLD, 3,400 PEG-$(cGMP)_2$, exhibiting a much higher apparent affinity and a lower Hill coefficient than values exhibited by cGMP, the short PLD, or the long PLD (17). However, unlike the situation in OLF channels, all of the PLDs were only partial agonists of RET channels, such that saturating concentrations activated <75% of the current activated by saturating cGMP. It was also noted that the control PLM was a full agonist. These observations suggest that when both cGMP moieties in the PLD bind to the RET channel, the polymer chain interferes either with full activation of the channel, or with ion permeation through the pore.

Results from kinetic experiments also support the notion that PLDs simultaneously occupy two binding sites. The effective dissociation rate of a PLD simultaneously bound to two sites on a protein, $k_{off(eff)}$ is given as

$$k_{off\,(eff)} = 2k_{off} * K_d/([C_{eff}]+K_d),$$

where k_{off} is the intrinsic off-rate of cGMP from a binding site, $[C_{eff}]$ is the effective concentration of cGMP at the second site, and K_d is the normal equilibrium dissociation constant of cGMP. In the case of dissociation of 2,000 PEG-$(cGMP)_2$ from the OLF channel, $[C_{eff}]$ is predicted to be 13.4 mM and the apparent K_d for cGMP is 4 mM, yielding an effective PLD dissociation rate that is 3,400-fold slower that for cGMP. Figure 3A shows that this intermediate length PLD did indeed exhibit a dramatically slowed dissociation rate as compared to cGMP or a very short PLD, 282 PEG-$(cGMP)_2$. In fact, after exposure to this intermediate PLD, the channels remained open for minutes even with continuous superfusion with agonist-free solution. Apparently, when one of the cGMPs in the PLD dissociates from the channel, the probability is high that it will re-bind

before its partner has an opportunity to dissociate. The off-rate of a longer PLD, 20,000 PEG-$(cGMP)_2$, was also slowed, but much less dramatically, consistent with the lower effective concentration of cGMP predicted for this molecule. Thus, it is likely that the decrease in effective dissociation rate accounts for the enhanced apparent affinity exhibited by PLDs for the OLF channel.

The dissociation of a ligand from a single binding site is normally unaffected by the concentration of free ligand in the bath solution. However, since PLDs appear to bind to 2 sites at once, occupancy of either of these sites with a competing ligand should speed the dissociation of the PLD. Figure 3B shows that when channels were first "loaded" with a PLD, subsequent application of cGMP did indeed accelerate the off-rate. A very high concentration (<1 mM) was required, however, because free cGMP had to compete with ligands on the PLD that were effectively present at concentrations of several millimolar. The observation that addition of free ligand (cGMP) can increase the dissociation rate of a bound ligand (the PLD) provides strong evidence that the PLD simultaneously occupies two sites on the channel.

While CNG channels all appear to have four nucleotide binding sites, the molecular dimensions may differ, including the distance between binding sites. To probe this distance, a series of PLDs was used, containing PEG polymers with molecular weights from 282 to 20,000, corresponding to rms lengths of 15 - 123 Å (12, 13, 18). Figure 4A shows that the PLD optimal for activating the RET CNG channels is shorter than the PLD optimal for activating OLF channels. Thus, 1,200 PEG-$(cGMP)_2$ (rms length of 30 Å) is best for activating RET, while 2,000 PEG-$(cGMP)_2$ (rms length of 39 Å) is best for activating OLF, suggesting that the cyclic nucleotide-binding sites are about 9 Å farther apart in OLF than they are in RET. Rather than exhibiting an abrupt increase at the optimal length, the apparent affinity of PLDs begins to increase before the optimal length is reached. This gradual increase in affinity is likely to result from elasticity in the PLD, although elasticity in the CNG channel may also contribute. Thus, even though an individual PLD may have an rms length somewhat shorter than optimal, it still has some finite probability of reaching the length required to span two binding sites. Using polymers that are "stiffer" than PEG, it may possible to generate PLDs that have little elasticity, and therefore are more finely tuned to specific proteins.

Identification of the optimal PLDs provides new information about the structure of CNG channels. The apparently wider spacing of binding sites in OLF is consistent with previous studies showing that the pore of the olfactory CNG channel is wider than the pore of the rod photoreceptor CNG channel (19). In contrast, the apparent distance between ligand binding sites (30-39 Å) indicated for CNG channels is shorter than the diameter of the acetylcholine receptor (65 Å), where structural measurements indicate that the putative ACh binding sites are located in cavities accessible from the outer perimeter of the extracellular portion of the receptor (20). Since CNG channels and acetylcholine receptors are similar in molecular weight, the closer spacing of cyclic nucleotide binding sites on CNG channels may indicate that sites on these channels are not located on the perimeter of the protein, but perhaps centered on the cytoplasmic surface of each subunit, as illustrated in Figure 1B.

The effect of the PLDs on PKG (21) was also examined. PKG is a homodimer, and each subunit has a regulatory (R) domain that contains two heterologous binding sites: one with high and one with low affinity for cyclic nucleotides. The R domain of PKG has extensive sequence homology with the R subunit of PKA (7), where structural determinations using X-ray crystallography indicate that the two sites are separated by 26 Å (22). Thus the two heterologous sites within a PKG monomer are also likely to be about 26 Å apart, while the distance between homologous cGMP-binding sites across two subunits is not known. Incubation of PKG with PLDs show that the dimer with the highest apparent affinity is the smallest PLD, 282 PEG-$(cGMP)_2$ (Fig. 4B). This PLD activated PKG with a 30-fold higher apparent affinity than did cGMP ($K_{1/2}$ = 4 nM for the PLD, K1/2 = 120 nM for cGMP), and 275-fold higher than did the PLM ($K_{1/2}$ = 1.1 mM) (23). The heightened apparent affinity of 282 PEG-$(cGMP)_2$ suggests that it does indeed simultaneously occupy two sites on PKG. However, the small expected rms length of this molecule implies that PKG activation does not result from the PLD binding to heterologous sites within a single PKG subunit. Rather, they suggest that PKG activation by the PLD primarily involves binding between subunits, with cyclic nucleotide binding sites less than 20 Å apart from one another.

For all three of the cyclic nucleotide-activated proteins that were examined (RET, OLF and PKG), at least some PLDs had apparent binding affinities that were dramatically

higher than cGMP. Indeed, the sensitivity of CNG channels for specific PLDs was nearly three orders of magnitude higher than for cGMP, making these molecules the most potent activators yet identified. The optimal PLD was different for each of the proteins, with 282, 1,200, and 2,000 PEG-$(cGMP)_2$ being optimal for PKG, RET, and OLF, respectively. Hence the PLD strategy appears to be useful for developing ligands that are not only highly potent, but also selective for specific proteins. Indeed, these studies have produced the first cyclic nucleotide derivative (2,000 PEG-$(cGMP)_2$, see Fig. 4) that activates at least some CNG channels (OLF) at a lower concentration than is required to activate PKG.

The present invention also provides PLDs that contain cyclic nucleotides other than cGMP, including derivatives such as Rp-cAMPS and Rp-cGMPS, that act as antagonists for specific cyclic nucleotide-binding proteins. By choosing the appropriate ligand composition of the PLD, and by selecting a specific size polymer, the invention may be used to provide highly effective antagonists of individual cyclic nucleotide-binding proteins. Moreover, at least some PLDs appear to be membrane-permeant, as determined in experiments in which extracellular application of 2,000 PEG-$(cGMP)_2$ results in activation of CNG channels in intact cells (data not shown). Hence specific PLDs may be useful for distinguishing whether physiological responses involving cGMP are mediated either by CNG channels or by PKG.

The multivalent tethered ligand approach has several remarkable features. Selective ligands can be synthesized and identified for different proteins that have similar or even identical ligand binding sites. Moreover there is no need for prior structural information about the target proteins. The high potency and selectivity of exemplary PLDs for closely related proteins indicates that this approach to drug design can be applied to many families of proteins that contain multiple binding sites, including extracellular and intracellular ligand-gated ion channels, transport proteins, and allosteric enzymes.

DISCLOSURES AND NOTES CITED BY NUMBER

1. J.T. Finn, M.E. Grunwald, K.-W. Yau, Annu. Rev. Physiol. 58, 395 (1996); W.N. Zagotta and S.A. Siegelbaum, Annu. Rev. Neurosci. 19, 235 (1996).

2. S.H. Francis and J.D. Corbin, Adv. Pharm. 26, 115 (1994).

3. S.S. Taylor, J.A. Beuchler, W. Yonemoto, Annu. Rev. Biochem. 59, 971 (1990).

4. U.B. Kaupp et al., Nature 342,762 (1989).

5. R.S. Dhallan, K.-W. Yau, K. Schrader, R. Reed, ibid. 347, 184 (1990).

6. Native CNG channels appear to be heterotetramers containing 2 a (4,5) and 2 b [T.-Y., Chen, Y.-W Peng,., R.S., Dhallan, B. Ahamed, R.R. Reed, K.-W. Yau., Nature 362, 764 (1993); E.R. Liman and L.B. Buck Neuron 13, 611 (1994); J. Bradley, J. Li, N. Davidson, H.A. Lester, K. Zinn Proc. Natl. Acad. Sci. USA 91, 8890 (1994); H.G. K"rschen, et al., Neuron 15, 627 (1995)] subunits, each containing a cyclic nucleotide binding site [R.L. Brown, R. Gramling, R.J. Bert, J.W. Karpen, Biochemistry 34, 8365 (1995)]. Homotetrameric channels composed solely of a-subunits can also be functionally expressed in Xenopus oocytes, and also contain 4 binding sites [D.T. Liu, G.R. Tibbs, S.A. Siegelbaum, Neuron 16, 983, (1996)].

7. J.M Shabb, and J.D. Corbin, J. Biol. Chem. 267, 5723 (1992).

8. L.H.P. Botelho, J.D. Rothermael, R.V. Coombs, B. Jastorff, Methods Enzymol. 159, 159 (1988); E. Butt, M. Van Bemmelen, L. Fischer, U. Walter, B. Jastorff, FEBS Lett. 263, 47 (1990); E. Butt, M. Eigenthaler, H.G. Genieser, Eur. J. Pharmacol. 269, 265 (1994); R.H. Kramer, and G.R. Tibbs, J. Neurosci. 16, 1285 (1996); J-Y. Wei, E.D. Cohen, Y.-Y. Yan, H-G. Genieser, C.J. Barnstable, Biochemistry 35, 16815 (1996).

9. R.L. Brown, R.J. Bert, F.E. Evans, and J.W. Karpen, Biochemistry 32, 10089 (1993).

10. PLDs were synthesized by reacting 8-thio cGMP (9) with bifunctional PEG vinylsulfone (VS-PEG-VS) (Shearwater Polymers, Inc, Huntsville, AB) at 37°C and pH 7.5 for 24-48 hours. PLDs were purified by reverse phase HPLC using a preparative C18 column eluted with a methanol gradient. Reactions were run either with an excess of 8-thio cGMP, to bias formation of PLDs (cGMP-PEG-cGMP), or with an excess of VS-

PEG-VS, to bias formation of half-reacted monomers (cGMP-PEG-VS). To distinguish between these products, for isolation of PLDs, the elution profiles of these two reactions were compared at 274 nm, a wavelength characteristic of the absorbance of thio-substituted cyclic nucleotides. The composition of 282 PEG-$(cGMP)_2$ was confirmed by mass spectrometry. The concentration of each PLD was determined by absorbance, assuming an extinction coefficient of 35,400 M^{-1} cm^{-1} (17,700 M^{-1} cm^{-1} for the PLM) (see 9).

11. A.L. Zimmerman, G. Yamanaka, F. Eckstein, D.A. Baylor, L. Stryer, Proc. Natn. Acad. Sci. USA 82, 8813 (1985); A. Caretta, A. Cavaggioni, R.T. Sorbi, Eur. J. Biochem. 153, 49 (1985); K.-W. Koch and U.B. Kaupp, J. Biol. Chem. 260, 6788 (1985); J.C. Tanaka, J.F. Eccleston, R.E. Furman, Biochemistry 28, 2776 (1989).

12. D. Knoll and J. Hermans, J. Biol. Chem. 258, 5710 (1983).

13. P. J. Florey Principles of Polymer Chemistry (Cornell University Press, Ithaca, New York, 1953).

14. Effective concentration (C_{eff}) is calculated by assuming the cognate cGMP is freely diffusible in a sphere with radius r, equal to the rms length of the PLD. Thus $C_{eff}=1{,}000/(N_A*4/3\pi r^3)$, where N_A is Avagodro's number (6.023×10^{23}), C_{eff} is expressed in units of molar, and r is in cm. If diffusion of the cognate cGMP is limited to a hemisphere, C_{eff} will be twice as large.

15. A "capped" PEG (5,000 MW) with a single VS group (methoxy-PEG-VS) was used to produce a control PLM.

16. Homomeric channels containing a-subunits of the bovine rod (4) or the rat olfactory CNG channel (5) were expressed in *Xenopus* oocytes. Borosilicate glass patch pipettes containing standard saline (in mM, 115 NaCl, 1 EDTA, 5 EGTA, 5 HEPES, pH 7.5) were used to obtain excised inside-out patches. The membrane potential was held at -75 mV in all experiments. cGMP and its PLD and PLM derivatives were prepared in standard saline and continuously superfused over excised patches at 21-23 °C.

17. For 3,400 PEG-$(cGMP)_2$, $K_{1/2}$=4.7 mM; n=0.9. For cGMP, $K_{1/2}$=72 mM; n=2.0. For 282 PEG-(cGMP)2, $K_{1/2}$=11 mM; n=1.3. For 20,000 PEG-$(cGMP)_2$, $K_{1/2}$>75 mM.

18. Since the derivatized PEGs used to synthesized PLDs exhibited polydispersity, each PLD was not a discrete molecular species, but rather a range of PLDs distributed around a mean value of MW. HPLC analysis showed that PLDs synthesized from a 1,000 MW PEG-(VS)2 contained a range of molecules with 15-33 ethylene glycol units, with a median at 24 units. HPLC fractions were collected to purify less polydispersed products with MW of 800 ± 132 (15-21 units) and 1,200 ± 132 (24-30 units). Similar degrees of polydispersity were exhibited by higher MW PLDs (2,000 - 20,000 MW). The 282 MW PLD exhibited no polydispersity because it was synthesized from a discrete derivatized PEG (6 ethylene glycol units). The two VS couplings added to the PEGs are likely to add a few Å to the rms length of all the PLDs.

19. E. H. Goulding, G.R. Tibbs, D. Liu, S.A. Siegelbaum, Nature 364, 61 (1993).

20. N. Unwin, J. Molec. Biol. 229, 1101 (1993); N. Unwin, Nature 373, 37 (1995).

21. PKG activity was assayed by determining incorporation of ^{32}P from γ-[^{32}P]-ATP into a heptapeptide substrate [K.R. Sekhar, et al., Mol. Pharmacol. 42, 103 (1992)]. Bovine recombinant PKG type 1 a was obtained from Calbiochem.

22. Y. Su, W.R. Dostmann, F.W. Herberg, K. Durick, N.H. Xuong, L. Ten Eyck, S.S. Taylor, K.I. Varughese, Science 269, 807 (1995).

23. The PLM was a partial agonist. At saturation, it activated about 30% of the PKG activity elicited by saturating cGMP.

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All books, articles and patents cited in this specification are incorporated herein by reference in their entirety.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the present invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus, it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the claims below.

CLAIMS:

1. A compound which is a multimeric tethered ligand comprising a plurality of ligand moieties tethered to a joint moiety, wherein each ligand moiety comprises a ligand for at least one receptor protein having multiple binding sites for said ligand and each ligand moiety is linked by a tether moiety to said joint moiety, said compound having a structure of the formula:

$$J(C_J^1\text{-}T^1\text{-}C_L^1\text{-}L^1)(C_J^2\text{-}T^2\text{-}C_L^2\text{-}L^2)(C_J^3\text{-}T^3\text{-}C_L^3\text{-}L^3)\ldots(C_J^x\text{-}T^x\text{-}C_L^x\text{-}L^x)$$

wherein:

x is the number of ligand moieties in said compound and is an integer greater than or equal to 2;

each of L^1 to L^x is one of said ligand moieties and comprises a ligand which is the same as or different from the ligand of any other ligand moiety in said compound,

J is said joint moiety selected from the group consisting of a covalent bond, an atom and a molecule;

each of C_J^1 to C_J^x is a joint coupling moiety selected from the group consisting of a covalent bond, an atom and a molecule, and is covalently coupled to said joint moiety;

each of C_L^1 to C_L^x is a ligand coupling moiety selected from the group consisting of a covalent bond, an atom and a molecule, and is covalently coupled to one of said ligand moieties, L^1 to L^x, respectively;

each of T^1 to T^x is a tether moiety and is a linear polymeric molecule having one end covalently coupled to said joint coupling moiety,C_J^1 to C_J^x, respectively, and the other end covalently coupled to a ligand coupler moiety, C_L^1 to C_L^x, respectively, thereby linking said ligand moiety to said joint moiety; and

each ligand moiety, L^1 to L^x, and tether moiety coupled thereto, T^1 to T^x, respectively, are not both peptides and are not both polynucleotides.

2. A compound of claim 1 wherein x = 2 and each of L^1 and L^2 comprises a ligand for the same receptor protein.

3. A compound of claim 2 wherein each of L^1 and L^2 comprises the same ligand.

4. A compound of claim 2 wherein each of T^1 and T^2 is a polymeric molecule selected from the group consisting of polymers containing only C, H and O, polynucleotides and polypeptides.

5. A compound of claim 4 wherein C_J^1,T^1, C_J^2 and T^2 together are a molecule of polyethylene glycol.

6. A compound of claim 5 wherein each of L^1 and L^2 is selected from the group consisting of compounds comprising a nucleoside, cyclic nucleotides, oligonucleotides, compounds comprising an amino acid, peptides, polypeptides, proteins, hormones, growth factors, cytokines, anti-idiotypic antibodies, small molecule ligands, drugs and metabolites.

7. A compound of claim 5 wherein each of L^1 and L^2 is a cGMP molecule or a derivative thereof, and each of C_L^1- and C_L^2-has the structure: S-CH_2-C H_2-SO_2- and is covalently coupled to the carbon in the 8 position of the guanosine moiety in said cGMP molecule or derivative thereof.

8. A compound of claim 7, wherein L^1 and L^2 are ligands for a receptor protein selected from the group consisting of a cyclic nucleotide-gated ion channel or a guanosine 3',5'-cyclic monophosphate activated kinase.

9. A compound of claim 8, wherein the affinity of said compound for said receptor is at least about 10 times to about 2,000 times greater than the average affinity of a monomer of said ligand for said receptor.

10. A method of activating or antagonizing activation of a receptor protein comprising multiple binding sites for a ligand L comprising the steps of:

adding to said receptor protein, under conditions where said ligand L activates or antagonizes activation of said receptor protein, a compound of claim 1 wherein each of said ligand moieties L^1 to L^x comprises said ligand L or a derivative thereof, and the average (rms) length of the portion of said compound linking any two of said ligand moieties L^1 to L^x is greater than or equal to the distance between any two of said multiple binding sites,

whereby each of at least two of said ligand moieties L^1 to L^x binds to one of said multiple binding sites on said receptor protein, thereby activating said receptor protein.

11. A method of obtaining a compound of claim 1 which activates or antagonizes activation of a receptor protein comprising multiple binding sites for a ligand L, said compound having a higher specific activity than said ligand L for activation of said receptor protein, said method comprising:

(a) providing a group of compounds of claim 1, each compound in said group having each of said ligand moieties L^1 to L^x comprising said ligand L or a derivative thereof, and each compound in said group differing in the average (rms) length of the portion of said compound linking any two of said ligand moieties L^1 to L^x, the average (rms) lengths of said portions in said molecules being selected to span the range of possible distances between any two of said multiple binding sites on said receptor protein,

(b) adding a fixed amount of each compound in said group separately to said receptor protein, under conditions where said ligand L activates or antagonizes activation of said receptor protein,

(c) determining the level of activation or antagonization of activation of said receptor protein produced by said fixed amount of each compound in said group,

(d) comparing the level of activation or antagonization of activation of said receptor protein produced by said fixed amount of each compound in said group to the level of activation or antagonization of activation obtained by adding the same fixed amount of said ligand L to said receptor under the same conditions, and

(e) selecting the compound in said group producing the highest level of activation or antagonization of activation of said receptor protein that is greater than the level of activation or antagonization of activation of said receptor by said ligand L.

12. A method of determining whether a receptor protein activated or antagonized by a ligand L comprises multiple binding sites for said ligand L such that binding of said ligand L to more than one of said multiple binding sites activates or antagonizes activation of said receptor protein to a higher level than does occupation of only one of said multiple binding sites, said method comprising:

(a) providing a group of compounds of claim 1, each compound in said group having each of said ligand moieties L^1 to L^x comprising said ligand L or a derivative thereof, and each compound in said group differing in the average (rms) length of the portion of said compound linking any two of said ligand moieties L^1 to L^x, the lengths of said portions in said molecules being selected to span the expected range of possible distances between any two of said multiple binding sites on said receptor protein,

(b) adding a fixed amount of each compound in said group separately to said receptor protein, under conditions where said ligand L activates or antagonizes activation of said receptor protein, and

(c) determining whether any compound in said group produces a higher level of activation or antagonization of activation of said receptor protein than produced by adding the same fixed amount of said ligand L to said receptor under the same conditions,

wherein a higher level of activation or antagonization of activation of said receptor protein by a compound in said group indicates that said receptor protein activated by ligand L comprises multiple binding sites for said ligand L such that binding of said ligand L to more than one of said multiple binding sites activates said receptor protein to a higher level than does occupation of only one of said multiple binding sites.

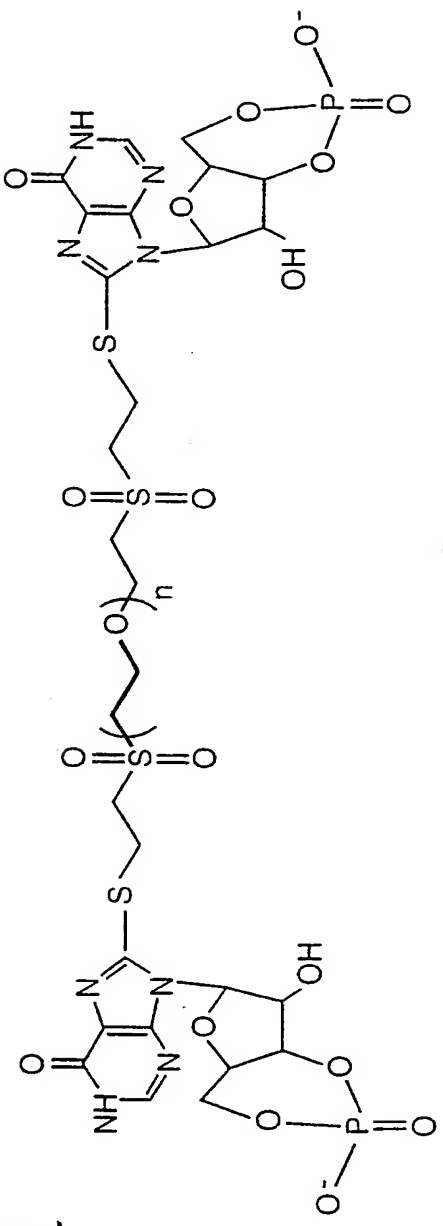


Fig. 1A

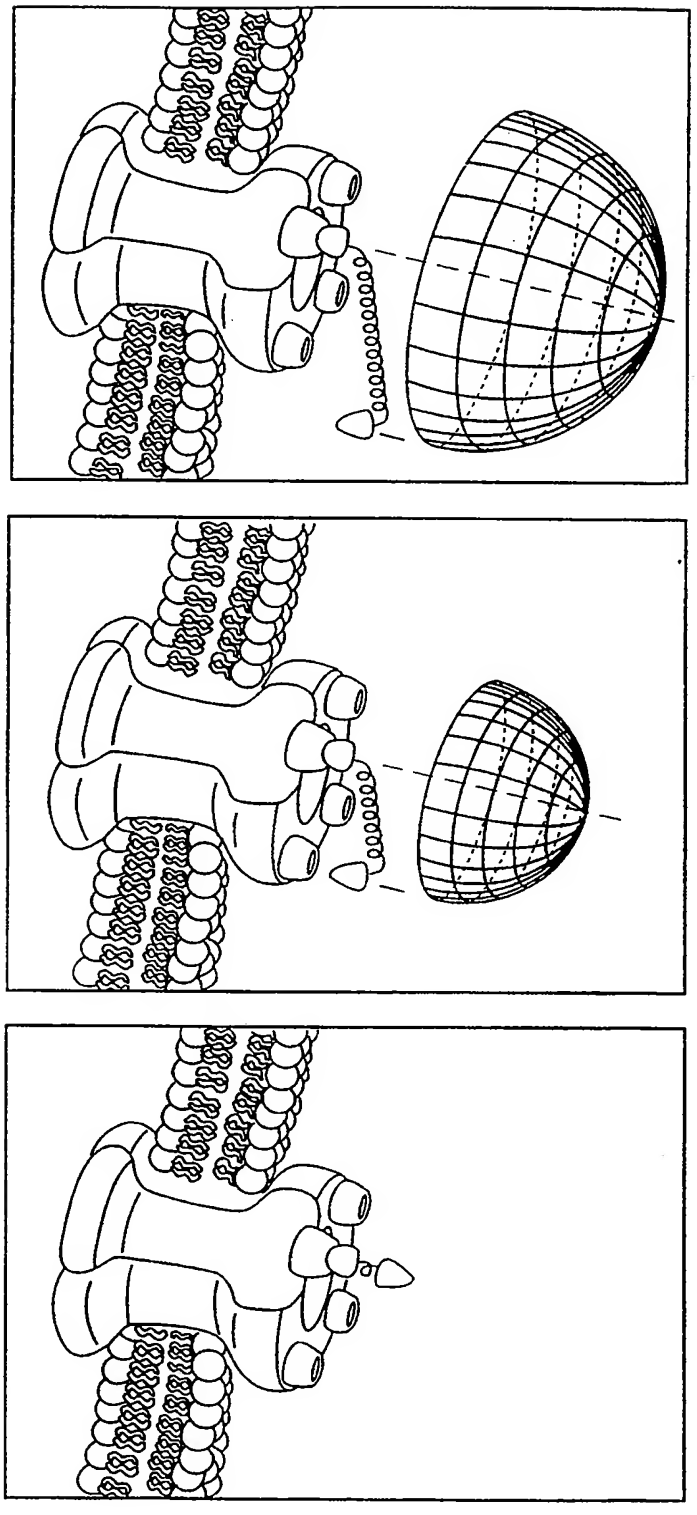


Fig. 1B

Fig. 2A

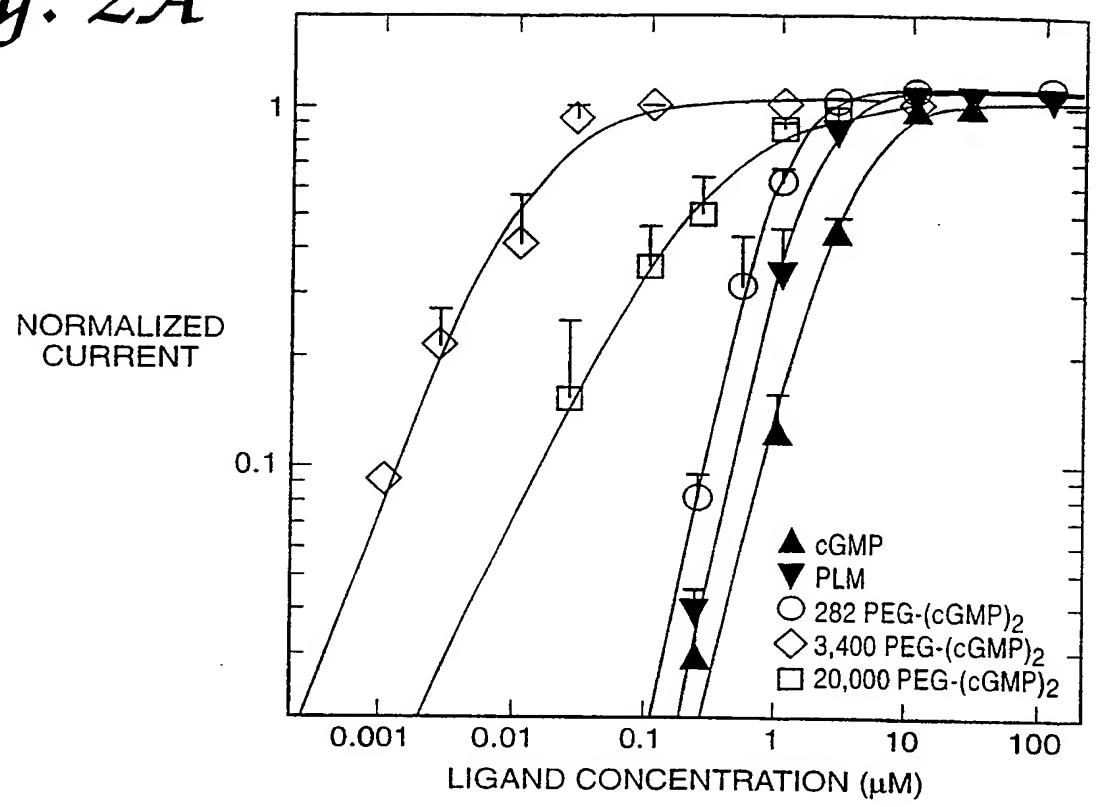


Fig. 2B

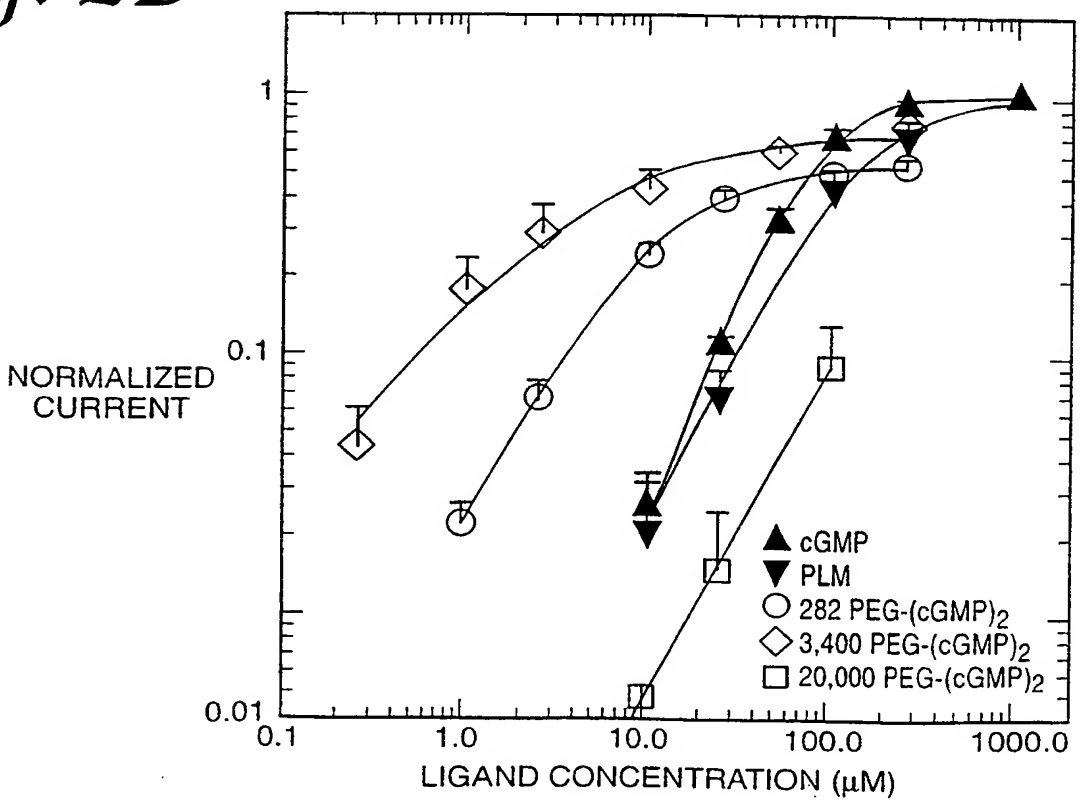


Fig. 3A

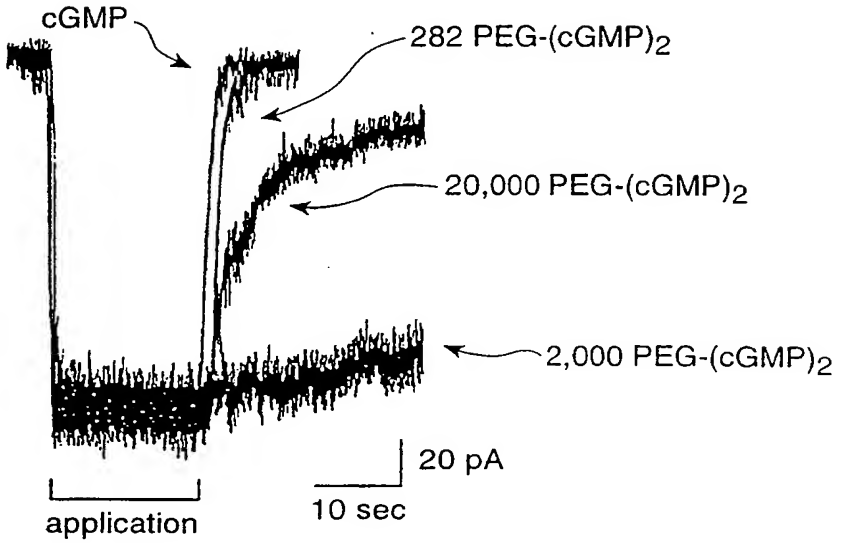


Fig. 3B

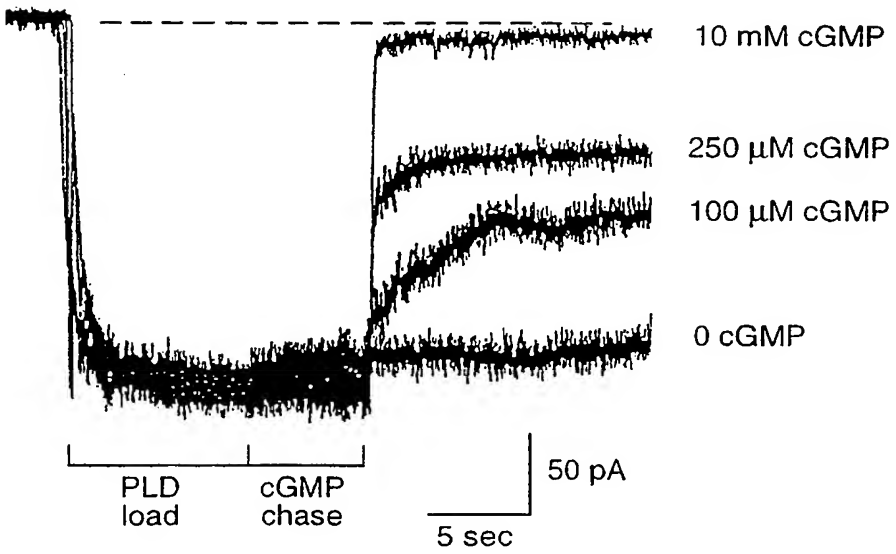


Fig. 4A

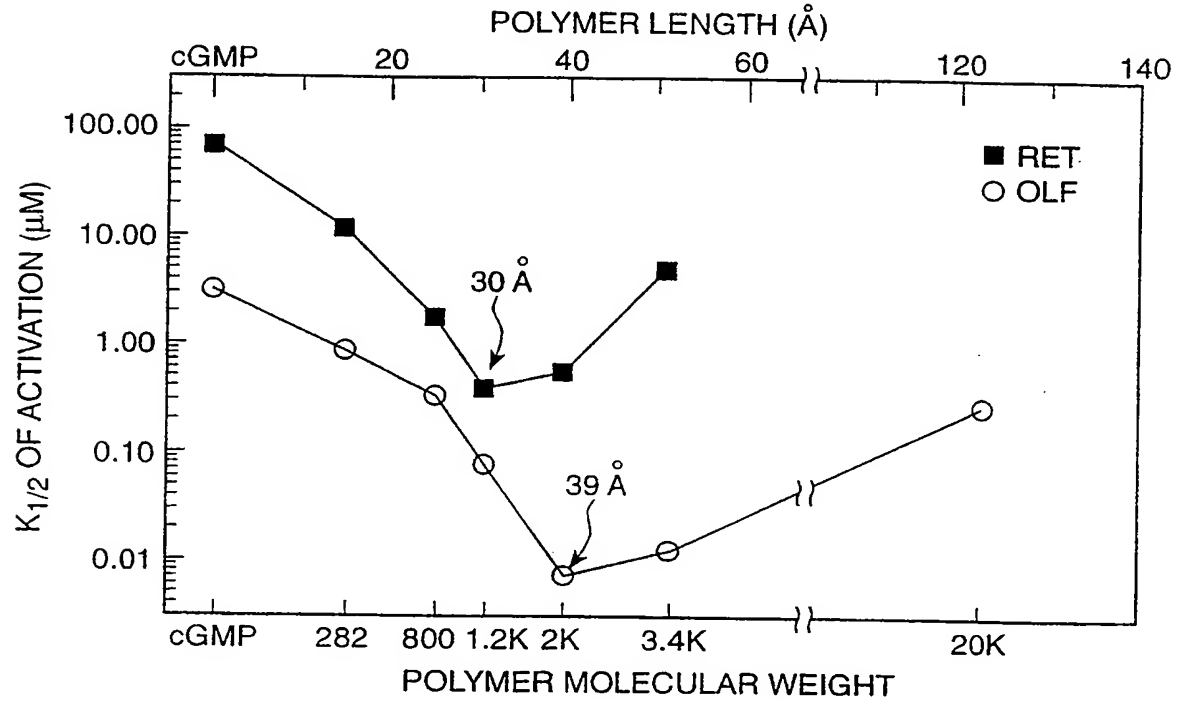
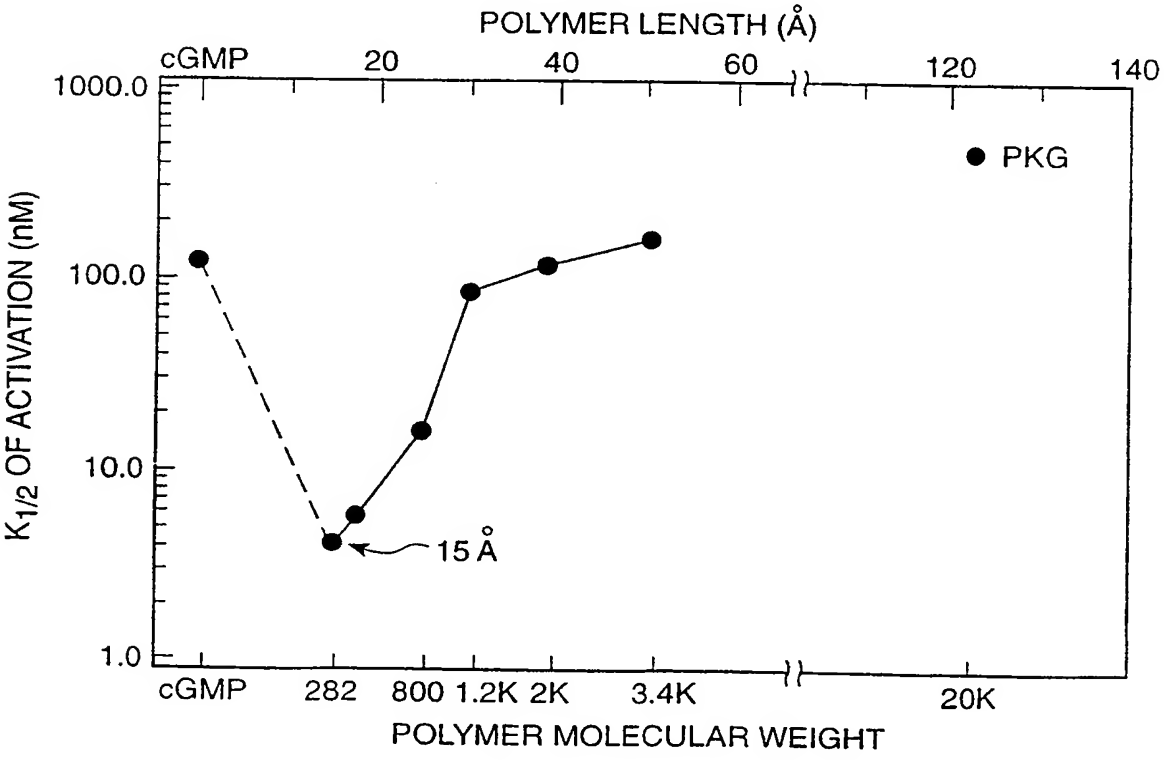


Fig. 4B



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(71) Applicants: UNIVERSITY OF MIAMI [US/US]; 1400 N.W. 10th Avenue, Miami, FL 33101 (US). UNIVERSITY TECHNOLOGY CORPORATION [US/US]; Suite 250, 3101 Iris Avenue, Boulder, CO 80301 (US).

(72) Inventors: KRAMER, Richard, H.; 5 Island Avenue #3C, Miami Beach, FL 33139 (US). KARPEN, Jeffrey, W.; University of Colorado Medical School, Dept. of Physiology and Biophysics, C240, 4200 East 9th Avenue, Denver, CO 80262 (US).

(74) Agents: LESTER, Michelle, N. et al.; Pillsbury Madison & Sutro, LLP, 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(57) Abstract

Multimeric tethered ligands are disclosed, comprising a plurality of ligand moieties tethered to a joint moiety, wherein each ligand moiety comprises a ligand for at least one receptor protein having multiple binding sites for the ligand and each ligand moiety is linked by a tether moiety to that joint moiety. Exemplary dimeric tethered ligands are disclosed in which the tether is a simple polymer, polyethylene glycol, which are therefore called "polymer–linked dimers" (PLDs). Exemplary PLDs containing two guanosine 3', 5' cyclic monophosphate (cGMP) moieties are described which are up to 1,000–fold more potent than cGMP in activating cyclic nucleotide–gated (CNG) channels and protein kinases. Each protein responds optimally to a PLD with a different average polymer length, indicating that each has a unique spacing of binding sites. Since optimal ligands are selected empirically, the multivalent tethered ligand strategy allows identification of highly potent and specific agents with no prior structural information about target proteins.

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KRAMER R H ET AL: "Spanning binding sites on allosteric proteins with polymer-linked ligand dimers." NATURE, (1998 OCT 15) 395 (6703) 710-3. JOURNAL CODE: NSC. ISSN: 0028-0836., XP002103102 ENGLAND: United Kingdom see the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24467

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	KRAMER, R. H. (1) ET AL: "Spanning binding sites on cyclic nucleotide-gated channels and kinases with polymer-linked ligand dimers." BIOPHYSICAL JOURNAL, (FEB., 1998) VOL. 74, NO. 2 PART 2, PP. A125. MEETING INFO.: FORTY-SECOND ANNUAL MEETING OF THE BIOPHYSICAL SOCIETY KANSAS CITY, MISSOURI, USA FEBRUARY 22-26, 1998 ISSN: 0006-3495., XP002103103 see the whole document	1-12
X	WO 96 40772 A (JOHNSON & JOHNSON ;JOHNSON DANA L (US); ZIVIN ROBERT A (US)) 19 December 1996 * See scheme 1 * see page 18, line 15 - page 19, line 19	1-6, 10-12
Y	see claims 1,9,15-19,25-27	1-12
Y	SCHUCKER SUZANNE B.: "Discovering High Afinity Ligands for Proteins: SAR by NMR" SCIENCE, vol. 274, 1996, pages 1531-1534, XP002103104 cited in the application see the whole document	1-12
Y	HUBER M ET AL: "2,2'- Dithiobis (N-ethyl-spermine -5-carboxamide) is a high affinity, membrane-impermeant antagonist of the mammalian polyamine transport system." JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 NOV 1) 271 (44) 27556-63. JOURNAL CODE: HIV. ISSN: 0021-9258., XP002103105 United States see page 27556, column 1; figures 1,2	1-12
P,Y	MATHAI MAMMEN ET AL.: "Polyvalent Interactions in Biological Systems: Impications for Design and Use of Multivalent LIgands and Inhibitors" ANGEW. CHEM. INT. ED. , vol. 37, 1998, pages 2754-2794, XP002103106 see page 2770 - page 2771	1-12
P,Y	WO 98 03632 A (UNIV YALE) 29 January 1998 see page 1-4 see claims 1,2,8,13,19-25,30,31,42 see claims 43,51,59,60,65,73,74,79 see claims 85,86,91,97,98,118,122. see claim 123	1-12

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/24467

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LEBOULLUEC, KAREN L. ET AL: "Bivalent indoles exhibiting serotonergic binding affinity" BIOORG. MED. CHEM. LETT. (1995), 5(2), 123-6 CODEN: BMCLE8;ISSN: 0960-894X,1995, XP002103840 see the whole document	1-12
Y	HAHN E F ET AL: "Irreversible opiate agonists and antagonists. II. Evidence against a bivalent mechanism of action for opiate azines and diacylhydrazones." JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, (1985 DEC) 235 (3) 839-45. JOURNAL CODE: JP3. ISSN: 0022-3565., XP002103841 United States see figures 1,2	1-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9640772 A	19-12-1996	US 5767078 A	16-06-1998
		AU 6100796 A	30-12-1996
		CA 2228277 A	19-12-1996
		EP 0892812 A	27-01-1999
WO 9803632 A	29-01-1998	AU 3810797 A	10-02-1998